Journal of Entomology and Zoology Studies
Available online at www.entomoljournal.com
JEZS Journal of Entomology and Zoology Studies

E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2016; 4(1): 332-337

Received: 17-11-2015
Accepted: 19-12-2015

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DNA barcoding: Tool for assessing species identification in Reptilia

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Abstract
Reptiles are an important group of cold blooded vertebrates that have significant ecological and evolutionary significance. Species identification of reptiles is generally based on morphological characters. With the advancement of modern molecular techniques, the concept of DNA barcoding has gained global attention. Here we discuss the concept of DNA barcoding and its role in identification, molecular phylogenetic studies, biodiversity assessment and conservation of reptiles. Since very few barcoding studies have been conducted on reptiles till date, there is need for global barcoding initiative exclusively for reptiles.

Keywords: Biodiversity, Crocodilia, DNA Barcoding, Reptiles, Squamata, Sphenodontia, Testudines

1. Introduction

Reptiles are cold blooded vertebrates which have horny scales and dry skin. The reptiles had dominated the world during the Mesozoic era which was regarded as the golden era of reptiles. During this era the giant dinosaurs prevailed which became extinct in the late cretaceous period. Modern reptiles are categorized into four orders namely Crocodilia, Squamata, Testudines and Sphenodontia. Poaching, overexploitation, habitat loss, risk from exotic species, emerging diseases and effect of climate change are adversely affecting the reptile populations around the world [1-3]. In some cases, application of rodenticide is also causing their decline by causing secondary poisoning [4]. Besides these, road mortality is also one of the reasons for the decrease of vertebrates including the reptiles in our environment [5]. The reptiles play a very important role both as predator and prey in the ecosystem. In agriculture, reptiles are necessary for biological control of rodents and insect pests.

Taxonomic classification and identification of reptiles is a challenging task. Generally the reptiles are classified based on classical morphological approaches. In the last decade, the molecular concept of DNA barcoding has become a trend for identification and subsequent phylogenetic studies for different types of organisms ranging from the microbes to the mammals. The reptiles are no exception. In DNA barcoding, short standardized region of a gene is used as molecular marker for species identification. About 650 bp region from the 5' end of the *Cytochrome c oxidase* subunit 1 (COI) gene was suggested as the barcode for animals [6]. Figure 1 shows the location of COI gene in the mitochondrial genome. This gene has been successfully used for barcoding different vertebrate groups like Fishes [7-8], Amphibians [9] Reptiles [10], Birds [11] and Mammals [12] and also for different invertebrate groups like like Porifera [13],Cnidaria [14], Platyhelminthes [15], Nemathelminthes [16], Mollusca [17-19], Arthropoda [20-22], Echinodermata [23-24]. DNA barcoding has significant role is assessing marine biodiversity [25-26].

In order to coordinate DNA barcoding globally, The Consortium for the Barcode of Life (CBOL) and Barcode of Life Data System - (http://www.barcodinglife.org) were launched. International Barcode of Life Project (iBOL) has 4 central nodes, 9 regional nodes and 7 country nodes spanning across the globe. A summary of the DNA barcoding procedure is shown in the Fig. 2. In this review, we present the DNA barcoding scenario in different groups of reptiles.

DNA Barcoding Crocodilia

The order crocodilia includes crocodiles, alligators, caimans and gharials. Recently, a study based on both morphological and molecular methods was used to study the systematics of

African crocodiles [27]. A separate barcoding study indicated that African crocodiles show higher genetic divergence than previously supposed [28]. DNA Barcoding of Indian crocodiles showed the efficiency of this technique for species delimitation [29]. For forensic purposes, skin samples were used for molecular identification of crocodiles [30]. There were taxonomic controversies regarding the phylogenetic position of African slender snouted crocodile, *Crocodylus cataphractus*. Taxonomists had placed this species within the genus *Crocodylus* based on morphological characters. But recent molecular analysis revealed that it not a member of the genus *Crocodylus* or *Osteolaemus* [31]. A time calibrated species tree was constructed for *Crocodylus* which also revealed that there is higher diversity among *Crocodylus*. Another important finding of this study was that *Crocodylus* originated in Late Miocene Indo- Pacific from a common ancestor and spread to other parts of the globe [32].

DNA Barcoding Squamata

The order squamata includes snakes, lizzards and worm lizzards. In 1994 (long before the DNA barcoding concept was put forward in 2004), mitochondrial DNA was used to study phylogenetic relationship in a lizard *Sceloporus grammicus* [33]. A large scale DNA barcoding of reptiles including the squamata was done using samples collected from Madagascar - a biodiversity hotspot [10]. This study revealed several new species which is one of objectives of DNA barcoding and also highlighted the role of DNA barcoding in conservation. Barcoding studies of sqamate reptiles at the Comoros archipelago proved the efficiency of DNA barcoding in detecting even the isolated populations [34]. DNA barcoding of German hepatofauna showed a success rate of almost 100% species identification [35]. Confiscated snakes in Korea were identified by DNA barcoding [36]. Moreover, this technique is used to identify the venom samples [37].

A very interesting case relating to wild life forensic science was revealed with the confiscation of a snake - wine bottle from a bar in Bangalore, India. Initially, based on morphological characters the snake was identified as Indian cobra-*Naja naja*. But the subsequent barcoding analysis revealed that the snake was Indo-Chinese spitting cobra - *Naja siamensis* [38]. The utility of DNA barcoding in wild life forensics is noteworthy [39-40]. Cytochrome b DNA sequences were used for DNA barcoding of endangered snakes [41]. The location of Cytochrome b (Cyt b) which is adjacent to the D-Loop (Displacement loop) in the mitochondrial genome is shown in the Fig 1.

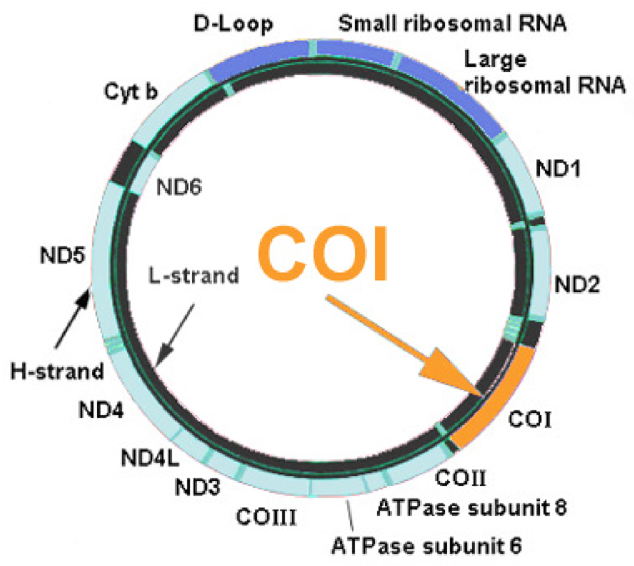

Fig 1: Position of COI gene in the mitochondrial genome

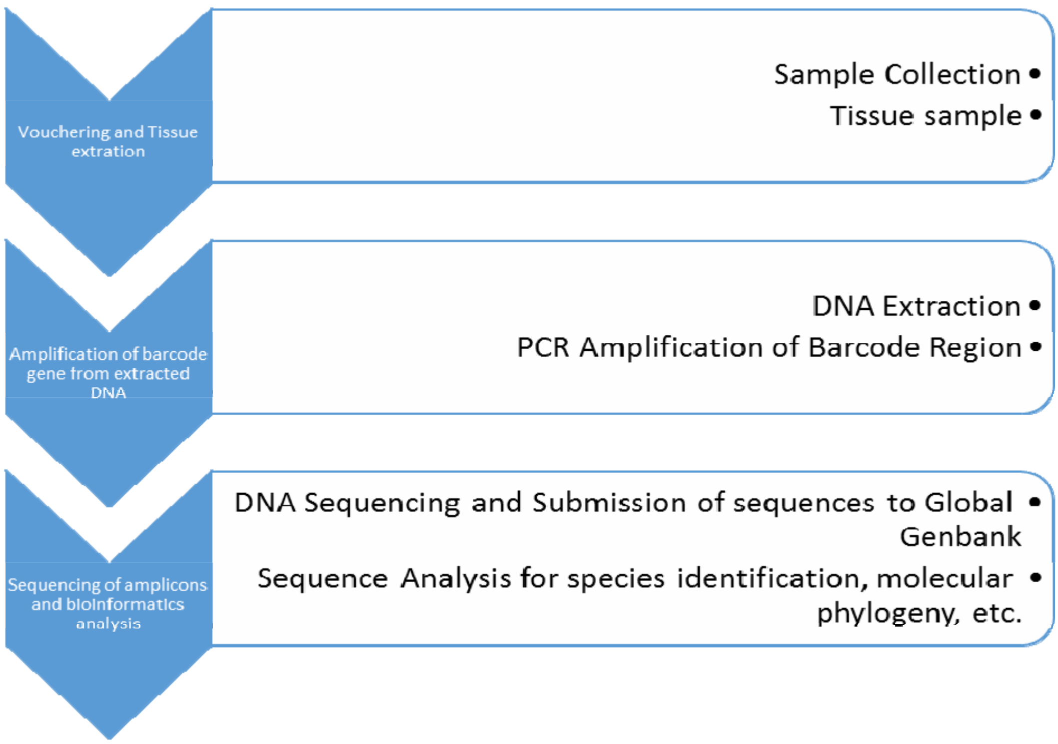

Fig 2: Summary of DNA barcoding Procedure

Table 1: Publications on DNA barcoding of reptiles

Group	Title of Article	Reference
Crocodilia	Rigorous approaches to species delimitation have significant implications for African crocodilian systematics and conservation.	Shirley *et al*. (2013)
	Are crocodiles really monophyletic?—Evidence for subdivisions from sequence and morphological data.	McAliley *et al*. (2006)
	Barcoding bushmeat: Molecular identification of Central African and South American harvested vertebrates.	Eaton (2010)
	Identification of Indian crocodile species through DNA barcodes.	Meganathan *et al*. (2013)
	A Time-calibrated species tree of *Crocodylia* reveals a recent radiation of the true crocodiles.	Oaks (2011)
Squamata	Comprehensive DNA barcoding of the herpetofauna of Germany	Hawlitschek *et al*. (2015)
	DNA barcode reference data for the Korean herpetofauna and their applications	Jeong *et al*. (2013)
	DNA barcoding assessment of genetic variation in two widespread skinks from Madagascar, Trachylepis elegans and T. gravenhorstii (Squamata: Scincidae)	Vences *et al*. (2014)
	DNA Barcoding Amphibians and Reptiles	Vences *et al*. (2012)
	First Large-Scale DNA Barcoding Assessment of Reptiles in the Biodiversity Hotspot of Madagascar, Based on Newly Designed COI Primers	Nagy *et al*. (2012)
	DNA-based identification of a snake in a wine bottle using universal primers: A case of mistaken identity.	Gaur *et al*. (2012)
	Forensic Wildlife Parts and their Product Identification and Individualization Using DNA Barcoding	Panday *et al*. (2014)
	Reliable DNA Barcoding Performance Proved for Species and Island Populations of Comoran Squamate Reptiles	Hawlitschek *et al*. (2013)
	Mitochondrial DNA sequences from dried snake venom: a DNA barcoding approach to the identification of venom samples	Pook and McEwing (2005)
	Application of cytrochrome b DNA sequences for the authentication of endangered snake species.	Wong *et al*. (2004)
	Mitochondrial DNA sequence divergence and phylogenetic relationships among eight chromosome races of the *Sceloporus grammicus* complex (Phrynosomatidae) in central Mexico	Arevalo *et al*. (1994)
Testudine	Taxonomic rank of Indian tortoise: revisit with DNA barcoding perspective.	Kundu *et al*. (2013a)
	Amino acid analysis of cytochrome c oxidase subunit 1(COI) of Indian testudines	Kundu *et al*. (2013b)
	Identification of commercialized turtle samples through DNA barcoding	Kundu *et al*. (2013c)
	DNA barcodes for globally threatened marine turtles: a registry approach to documenting biodiversity.	Naro-Maciel *et al*. (2009)
	The dazed and confused identity of Agassiz's land tortoise, *Gopherus agassizii*, the description of a new species, and its consequences for conservation.	Murphy *et al*. (2011)
	DNA barcoding of Brazilian sea turtles (Testudines).	Vargas *et al*. (2009)
	Phylogenetic relationship among extinct and extant turtles: the position of Pleurodira and the effects of the fossils on rooting crown-group turtles	Sterli *et al*. (2010)
	Comparing and combining distance-based and character-based approaches for barcoding turtles	Reid *et al*. (2011)
	Multiple data sets, high homoplasy, and the phylogeny of softshell turtles (Testudines: Trionychidae)	Engstrom *et al*. (2004)
	Mitochondrial DNA sequences suggest a revised taxonomy of Asian flapshell turtles (*Lissemys* Smith,1931) and the validity of previously unrecognized taxa (Testudines: Trionychidae)	Praschag *et al*. (2011)
Sphenodontia	A New Rhynchocephalian from the Late Jurassic of Germany with a Dentition That Is Unique amongst Tetrapods.	Rauhut *et al*. (2012)

DNA Barcoding Testudines

Testudines include turtles and tortoises. They are shelled reptiles. One of the main differences between turtles and tortoises is that turtles are aquatic but tortoises are terrestrial. Recent barcoding studies have shown that Agassiz´s desert tortoise *Gopherus agassizii* occupies only 30% of its originally proposed range and in the past 30 years there is drastic decline in their population. If proper conservation strategies are not implemented, this endangered species may face extinction [42].

A study on the sea turtles of Brazil showed that COI sequences can be efficiently used to barcode these species [40]. DNA Barcoding is very effective for identification, evaluation and conservation of threatened marine turtles [43]. The efficacy of this technique was also tested with several tortoise species of north east India [44]. Nuclear intron from the RNA fingerprint protein 35 was first time used to study turtle phylogeny [45]. Rhodin *et al*., [46], provided an updated checklist of the world turtles. Softshell turtles (Trionychids) are facing environmental and anthropogenic threat which is leading to the decline in its population [47]. Due to confusing morphological data in the different life cycle stages, it becomes very difficult to identify the softshell turtles. This is a serious hurdle for the taxonomists and conservation strategists. DNA barcoding provided clarity in the species discrimination of softshell turtles [48]. The ambiguities regarding the species identification of turtles and tortoises can be solved by DNA barcoding [49]. Cytochrome c oxidase subunit 1 (COI) is ideal mitochondrial marker for phylogenetic studies as it is largest among the three cytochrome oxidase subunits and amino acid sequences have highly conserved functional domains and variable sites [50].

Thereafter it has been revealed that along with the COI sequences, the amino acids informative sites within the COI region may play a vital role in discriminating the different testudine species [51]. This study on Indian testudines showed that first codon position had GC bias (54%) whereas the second and third codon positions had AT bias of 56.4% and 67.4% respectively. Furthermore, it was found that nucleotide variation and amino acid variation were found in 293 and 57 positions respectively.

DNA Barcoding Sphenodontia

Tautaras of New Zealand are included in the order Sphenodontia. Sphenodon is the only surviving genus belonging to the order Sphenodontia. It is speculated that they lived along with the dinosaurs and separated from other reptile groups more than 200 million years ago. A group of scientists regard them as living fossils. About a quarter of Sphenodon population have become extinct in the last century [52].

Scientists have reported that bad taxonomy can lead to serious decline of species [52-53]. Jones *et al*., [54] studied the pre-Pleistocene and post-Mesozoic rhynchocephalian fossil remains from the Miocene of New Zealand. This remains dating back 18 million years, has raised questions whether New Zealand was fully submerged some 25 million years ago as previously thought. A new fossil relative of Sphenodon from the latest Jurassic of southern Germany, Oenosaurus muehlheimensis has been described [55]. Scientists at Griffith University are working on the mitochondrial genome of Sphenodon.

Conclusion

Although reptiles represent significant vertebrate diversity, fewer barcoding studies have been conducted compared to other vertebrates. At present there is no global initiative for barcoding exclusively on reptiles. In contrast we have global barcoding initiatives for other vertebrates like Fish (FISH-BOL-http://www.fishbol.org), Birds (All Birds Barcoding Initiative - ABBI), Mammals (Mammalia Barcode of Life - http://www.mammaliabol.org). Our experience in the lab has shown that different sets of primers or cocktail of primers are needed for barcoding reptiles which makes barcoding of reptiles relatively difficult. This technical problem has been highlighted and subsequently new primer sets were suggested [10].

Recently, a global effort to barcode all cold-blooded vertebrates has been launched which is called 'Cold Code'. This initiative is expected to reveal important barcoding data on cold blooded vertebrates including reptiles [42]. Paleobarcoding is supposed to decipher important insights into the identification and phylogeny of ancient reptiles. With the advent of high-throughput DNA sequencing it is now possible to amplify mass collection of different species and even environmental DNA. Metabarcoding can shorten the timespan for identification procedure. With these advancements, DNA barcoding can serve as an important molecular tool for biodiversity assessment and conservation of reptiles.

Acknowledgements

We acknowledge the Deanship of Scientific Research, University of Tabuk, Saudi Arabia, for the support provided through the Project No. S-1436-0252. We also express our gratitude to the Saudi Digital Library (SDL).

Conflict of interest: No conflict of interest reported in the current work.

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